EXHIBIT A

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4′-Thio-oligo-β-D-ribonucleotides: synthesis of β-4′-thio-oligouridylates, nuclease resistance, base pairing properties, and interaction with HIV-1 reverse transcriptase

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ABSTRACT

We present the synthesis and the study of properties of a new series of modified oligonucleotides, namely 4′-thio-oligo-β-D-ribonucleotides (4′-S-RNA). Homo-oligonucleotides of this class (4′-SU_6 and 4′-SU_{12}) were prepared from the previously known thionucleosides using the phosphoramidite methodology. The comparison of the substrate properties of 4′-SU_6 and its natural analog U_6 with respect to four nucleases indicates that the former is much more resistant than the latter. Such resistance to nucleases in addition to relatively high Tm values for 4′-SU_{12} hybridized with Poly(A) show that these new 4′-S-RNA are good candidates for potential antisense effects. The oligonucleotides 4′-SU_6 and 4′-SU_{12} have been also evaluated as non sequence specific inhibitors of HIV-1 reverse transcriptase. All available evidences, based primarily on fluorescence measurements, are consistent with the binding of 4′-SU_6 and 4′-SU_{12} to RT at a site which is different from the polymerase site of the enzyme.

INTRODUCTION

Numerous structural modifications of oligonucleotides have been proposed to improve their stability towards enzyme degradation and/or to increase their binding affinity and specificity to complementary nucleic acids. The most noteworthy alterations concern the phosphate backbone, as exemplified by the methylphosphonate or phosphorothioate oligodeoxynucleotide series [1]. Such modified oligomers can be easily prepared from commercialy available β-deoxynucleosidic units. Oligodeoxynucleotide analogs with structural changes in the sugar moiety are less easily accessible since modified nucleosidic units must first be synthesized. However, α-oligodeoxynucleotides [2], carbaoligodeoxynucleotides [3], L-oligodeoxynucleotides [4], and oligo-2′-deoxyxylonucleotides [5] have been previously obtained.

On the other hand, oligoribonucleotides have been less well explored due to the more difficult synthetic approach imposed by the 2′-extra functionality. In this respect, apart from the α-RNA series [6, 7] or the 2′-O-substituted RNA series [8, 9], few other sugar modified oligoribonucleotides have been well described. However, RNA analogs have recently gained new interest due to the finding of catalytic RNA. For this reason, we have been interested in designing a new oligoribonucleotidic series with potential catalytic activity in antisense strategies and with eventual inhibitory effect on RNA binding enzymes or regulatory proteins. Such a new class of oligoribonucleotides must retain the natural β-D-ribonucleosidic configuration, the 2′-OH group available for catalysis and, in our opinion, the unmodified phosphate groups for solubility and mimicry of folded structure, and to avoid introduction of chirality. This led us to consider the isosteric replacement of the annular oxygen atom of the ribofuranose by a sulfur atom in the hope of improving nuclease resistance and binding affinities of the corresponding oligomers. This approach requires the preparation of the 4′-thio-nucleosides as starting synthons. Although known for about 25 years [10, 11], such nucleosides have been recently re-evaluated mainly as their deoxynucleosides derivatives for their potential antiviral properties by Walker [12] and Secrist [13]. However, the corresponding oligomers have not yet been prepared. As we were mainly interested in the oligoribonucleotide series, we first designed a new synthetic pathway for the 4′-thio-ribonucleosides [14] and, in preliminary experiments, we estimated the influence of introducing only one 4′-ST synthon in synthetics dT oligomers [15]. Since such modified oligonucleotides exhibited nuclease resistance while retaining Watson–Crick base pairing [15], we decided to synthesize new homogeneous 4′-thio-oligo-ribonucleotides (namely 4′-SU_6 and 4′-SU_{12}) in order to explore their stability towards nuclease degradation and their binding characteristics with complementary DNA and RNA strands. In addition, the interaction of these oligomers with HIV-1 reverse transcriptase was also studied in order to evaluate their eventual inhibitory capacity.

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MATERIALS AND METHODS

Materials

Calf spleen phosphodiesterase, snake venom phosphodiesterase, ribonuclease A and endonuclease S_1 were bought from Boehringer-Mannheim. Cell culture medium RPMI 1640 and fetal calf serum were purchased from GIBCO BRL.

[^{3}H]dTTP was obtained from Amersham, Poly(A) and dT_{14} from Sigma. Recombinant HIV-1 reverse transcriptase (RT) was expressed in *Escherichia coli* and purified as already published [16]. Highly homogeneous preparations of the RT heterodimer p66.p51 were used in all experiments.

General methods

Melting points were determined in a Buchi-Tottoli 510 apparatus and are uncorrected. Ultraviolet spectra (UV) were recorded on a Uvikon 810 spectrophotometer (Kontron). Optical rotations were measured in a 1 cm cell at 20°C in a Perkin-Elmer model 241 spectropolarimeter. ^{1}H-NMR spectra were recorded in a Bruker AC 250, AM 300 or WM 360 WB spectrometer in DMSO-d_6 or in CD_3CN. Chemical shifts (d) are quoted in parts per million relative to tetramethylsilane set at 0.0 ppm as internal reference. The signals are described as: s, singulet; d, doublet; t, triplet; dd, double doublet; m, multiplet. ^{31}P-NMR spectra were recorded in $CDCl_3$ on a Bruker WP 200 spectrometer with ^{1}H broad band decoupling. Chemical shifts are expressed downfield from external 66% H_3PO_4. Fast Atom Bombardment (FAB) mass spectra (m/z) were determined in the positive or negative ion mode on a JEOL DX 300 mass spectrometer. Thin-layer chromatography (TLC) was performed on pre-coated silica gel sheets 60 F_{254} (Merck, no. 5554) and column chromatography was performed with silica gel 60 (Merck no. 9385).

High performance liquid chromatographic (HPLC) analyses and purifications of 4'-thio-oligoribonucleotides were carried out on a SFCC C_{18} XLODS (3μm) column. The HPLC system (Waters Millipore) included a Model U6K injector, two Models 6000 pumps, a Model 680 gradient controller and a Model 990 photodiode array detector interfaced with a NEC APC IV computer. A linear gradient of 10−15% acetonitrile in 0.05 M aqueous solution of triethylammonium acetate (pH 7) was applied over 20 min at a flow rate of 1 ml.min^{-1}.

High performance liquid chromatographic (HPLC) analyses of enzymatic degradation of 4'-thio-oligomers were performed using the 'on line ISRP cleaning' method [17]. A linear gradient of 0 to 0.3 M KCl in 20 mM KH_2PO_4, 20% CH_3CN, pH 6 buffer was applied in 15 min at a flow rate of 1 ml.min^{-1}.

The fluorescence experiments were carried out using a SLM-Smart 8000 spectrofluorimeter equiped with a PH-PC 9625 photomultiplier, and with spectral bandwidths of 2 and 8 nm for excitation and emission, respectively. Measurements were performed at 25°C in a buffer containing 50 mM Tris−HCl pH 8.0, 10 mM $MgCl_2$, 50 mM KCl and 1 mM DTT. The total volume was 0.7 ml. All measurements were corrected for the wavelength dependence on the exciting light intensity through the use of Rhodamine B in the reference channel.

The steady state kinetic experiments were performed as already published [18]. The hybrids were prepared in 20 mM Tris, HCl pH 7.5 by mixing Poly(A) and the corresponding oligomer with a nucleotide number ratio of 5: 1 in favor of Poly(A); the solution was then heated at 65°C for 10 min then allowed to cool slowly. The concentrations of duplexes refer to the concentrations of the corresponding hybridized primers or primer analogs.

Oligoribonucleotide synthesis

Synthesis of ribonucleoside phosphoroamidite units

1-[5'-O-dimethoxytrityl -4'-thio-β-D-ribofuranosyl]-uracil **2**. This compound was prepared using standard procedure from 1-[4'-thio-β-D-ribofuranosyl]-uracil **1** [14] Yield 70%, (FAB>0, 3-nitrobenzyl-alcohol) m/z 563 [M+H]$^+$. ^{1}H-NMR (360 MHz, DMSO-d_6) δ 11.31 (s, 1H, NH), 7.70 (d, 1H, H_6, $J_{6,5}$ = 8.0), 7.40−6.89 (m, 1^3H, ArH), 5.84 (d, 1H, $H_{1'}$, $J_{1',2'}$ = 5.7), 5.53 (d, 1H, $OH_{2'}$, $J_{OH,2'}$ = 5.6), 5.48 (d, 1H, H_5, $J_{5,6}$ = 8.0), 5.26 (d, 1H, $OH_{3'}$, $J_{OH,3'}$ = 4.4), 4.02 (m, 2H, $H_{2'}$, $H_{3'}$), 3.74 (s, 6H, 2 OCH_3), 3.31 (m, ^{3}H, $H_{4'}$, $H_{5'}$, $H_{5''}$).

1-[5'-O-dimethoxytrityl -2'-O-tert-butyldimethylsilyl-4'-thio-β-D-ribofuranosyl]-uracil, **3**, *and 1-[5'-O-dimethoxytrityl -3'-O-tert-butyldimethylsilyl-4'-thio-β-D-ribofuranosyl]-uracil,* **4**. To a solution of 1-[5'-O-dimethoxytrityl-4'-thio-β-D-ribofuranosyl]-uracil **2** (0.562 g, 1 mmol) in anhydrous THF (10 ml), silver nitrate (0.203 g, 1.2 mmol) in dry pyridine (0.193 ml, 3.7 mmol) was added. This mixture was stirred at room temperature for 5 min and then *tert*-butyldimethylsilyl chloride (0.195 g, 1.3 mmol) was added. The reaction mixture was stirred at room temperature for 24 hr. The reaction mixture was then filtered, poured into 5% aqueous sodium hydrogencarbonate solution (30 ml) and the products were extracted with methylene chloride (3×20 ml). The combined organic layers were washed with water (2×50 ml), dried over sodium sulfate and evaporated to dryness under reduced pressure. The residue was applied to a silica gel column and chromatographed using a mixture of ethyl acetate in methylene chloride (5/95 to 20/80). The fast eluting fractions containing the 2'-O-TBDMS isomer **3** were collected. The slow eluting fractions containing mainly the 3'-O-TBDMS derivative **4** were dissolved in ethanol and stirred at room temperature overnight. The solvent was then evaporated to dryness. The residue was chromatographed as previously described. Repetition of this procedure three times allowed the recovery of additional pure 2'-*tert*-butyldimethylsilylated derivative that was lyophilized from dioxane to afford a colorless powder.

1-[5'-O-dimethoxytrityl -2'-O-tert-butyldimethylsilyl-4'-thio-β-D-ribofuranosyl]-uracil, 3. Yield 56%, mp 114−115°C. (FAB>0, 3-nitrobenzyl alcohol) m/z 677 [M+H]$^+$, 619 [(M+H-(*tert*-butane)]$^+$. ^{1}H-NMR (360 MHz, DMSO-d_6) d 11.20 (s, 1H, NH), 7.81 (d, 1H, H_6, $J_{6,5}$ = 8.1), 7.30−6.89 (m, 1^3H, ArH), 5.84 (d, 1H, $H_{1'}$, $J_{1',2'}$ = 5.5), 5.23 (d, 1H, $OH_{3'}$, $J_{OH,3'}$ = 4.7), 4.11 (dd, 1H, $H_{2'}$, $J_{2',1'}$ = 5.5, $J_{2',3'}$ = 3.5), 3.95 (m, 1H, $H_{3'}$), 3.74 (s, 6H, 2 OCH_3), 3.38 (m, 2H, $H_{4'}$, $H_{5'}$), 3.28 (m, 1H, $H_{5''}$), 0.82 (s, 9H, *tert*-butyl), 0.05 (m, 6H, $(CH_3)_2$Si).

1-(5'-O-dimethoxytrityl -3'-O-tert-butyldimethylsilyl-4'-thio-β-D-ribofuranosyl)-uracil, 4. Yield 27%, mp 109−110°C. (FAB>0, 3-nitrobenzyl-alcohol) m/z 677 [M+H]$^+$, 619 [(M+H)-(*tert*-butane)]$^+$. ^{1}H-NMR (360 MHz, DMSO-d_6) δ 11.30 (s, 1H, NH), 7.70 (d, 1H, H_6, $J_{6,5}$ = 8.0), 7.30−6.89 (m, 1^3H, ArH), 5.84 (d, 1H, $H_{1'}$, $J_{1',2'}$ = 7.30), 5.57 (d, 1H, H_5, $J_{5,6}$ = 8.0), 5.46 (d, 1H, $OH_{2'}$, $J_{OH,2'}$ = 5.1), 4.12 (t, 1H, $H_{3'}$, $J_{3',2'}$ = 3.1, $J_{3',4'}$ = 3.1), 4.03 (m, 1H, $H_{2'}$), 3.74 (s, 6H, 2 OCH_3), 3.41 (m, 1H, $H_{4'}$), 3.21 (m, 2H, $H_{5'}$, $H_{5''}$), 0.08 (s, 9H, *tert*-butyl), 0.02 (m, 6H, $(CH_3)_2$Si).

Table 1. Enzyme concentrations and enzymatic cleavage buffers used in the degradation experiments.

Enzyme	Enzymatic activity units / ml	Enzymatic cleavage buffer
Calf spleen phosphodiesterase	13.10^{-3}	EDTA 0.25 mM $AcONH_4^+$ 0,125 mM pH 7 Tween 80 0,0625 %
Snake venom phosphodiestearse	6.10^{-4}	Tris. HCl 0,1 N pH 9,4 $MgCl_2$ 0,01 M
Endonuclease S_1	20	AcONa 0,05 N NaCl 0,3 M pH 4.7 AcZn 0.1 M
Ribonuclease A	2.10^{-2}	NaCl 0,3 M Tris. HCl 0,1 M pH 7,4 EDTA 5 mM

1-[5'-O-dimethoxytrityl-2'-O-tert-butyldimethylsilyl-3'-O-(methoxy-N,N-di-isopropyl-phosphoroamidite-4'-thio-β-D-ribofuranosyl]-uracil, 5. To a solution of **3** (0.676 g, 1 mmol) in dry methylene chloride (3.8 ml) flushed with argon, N, N-di-isopropylethylamine (0.7 ml, 4 mmol), N, N-di-isopropylamino-methoxy-chloro-phosphine (0.48 ml, 2.5 mmol) and 4-dimethylaminopyridine (0.024 g, 0.2 mmol) were added. The reaction mixture was stirred at room temperature for 40 min and then diluted in ethyl acetate (35 ml), washed with brine (4×50 ml) and water (2×50 ml). The organic phase was dried over Na_2SO_4, filtered, concentrated, applied on a silica gel chromatography column and eluted with a mixture of cyclohexane/methylene chloride and triethylamine (v/v/v: 100/0/0.1 to 50/50/0.1). **5** was finally lyophilized in benzene and the mixture of diastereoisomers was obtained as a white powder (yield: 78%).(FAB<0, polyethyleneglycol) m/z 835 $[M-H]^-$. ^{31}P-NMR ($CDCl_3$), δ 150.65 and 150.50. ^1H-NMR (CD_3CN) δ 7.99 (s, 1H, NH), 7.22 (m, 1H, H_6), 7.30−6.89 (m, ^1H ArH), 5.90 (m, 1H, $H_{1'}$), 5.50 (m, 1H, H_5), 4.13 (m, 2H, $H_{2'}$, $H_{3'}$), 3.80 (s, 6H, 2 OCH_3), 3.58 (m, ^3H, $H_{4'}$, $H_{5'}$, $H_{5''}$), 3.42 (m, 2H, 2 $CH(Me)_2$), 3.30 (m, ^3H, OCH_3), 1.19 (m, 12H, 2 $(CH_3)_2CH$-), 0.84 (s, 9H, *tert*-butyl), 0.05 (m, 6H, $(CH_3)_2Si$).

Synthesis of the propanediol containing support, 6 ('Universalis' solid support)

This solid support was synthesized according to the already described procedure [4]. The amount of 1-O-dimethoxytrityl-3-propanol loaded onto the resin was 29.7 μmol/g as calculated from dimethoxytrityl cation released by 0.1 M *p*-toluene sulfonic acid in acetonitrile.

Functionalisation of the controlled pore glass solid support, 7

LCA-CPG beads bearing succinic acid arms were reacted with 1-[5'-O-DmTr-3'-O-TBDMS-4'-thio-β-D-ribofuranosyl]-uracil 4 according to the procedure described in reference 19. This solid support was finally obtained with a functionalisation state of 21 μmol/g.

Solid-phase synthesis of homo-oligoribonucleotides

4'-SU_6, **8**, 4'-SU_{12}, **9** and 4'-SU_6-nprOH, **10** (where nprOH designs a phosphodiester propanol linkage at the 3' termini of the oligomer) were synthesized on a Applied Biosystem 381A syntheziser following the standard procedure described for β-D-oligoribonucleotides [20], which requires an extended 15 min coupling step as compared to oligodeoxyribonucleotides. Individual syntheses were carried out on a column containing respectively 0.827 μmol (39.4 mg), 0.835 (39.8 mg) and 0.820 μmol (36.2 mg) of suitable solid supports **7** or **6**. After completion of the assembling, solid supports were treated with thiophenol-triethylamine-dioxane (v/v/v: 1/2/2, 0.5 ml) for one hour at room temperature and washed with methanol (10 ml). The solid supports were then reacted three times with concentrated (32%) aqueous ammonia-ethanol (v/v: 3/1, 0.5 ml) for 30 min at room temperature. The ammonia solutions were concentrated and 1.1 M *tetra*-butyl ammonium fluoride (0.5 ml) was added to the dry residues. After 24 hours at 20°C, the reactions were quenched with a 0.05 M aqueous ammonium acetate solution (0.3 ml) and evaporated to dryness. The residues were chromatographed on DEAE G-25 Sephadex and eluted with water. The main U.V. absorbing fractions were combined and purified on reverse phase HPLC (see General Methods) to afford finally 4'-SU_6 (7.4 A_{260}), 4'-SU_{12} (11.7 A_{260}) and 4'-SU_6-nprOH (1.2 A_{260}) with respective spectrophotometric purities of 98.3%, 98.8% and 98.0%.

Thermal melting experiments

Optical measurements were performed on a UVIKON 810 spectrophotometer (KONTRON) interfaced with an IBM PC compatible microcomputer. The temperature control was done with a HUBER PD 415 temperature programmer connected to a refrigerated ethylene glycol-water bath (HUBER Ministat). Cuvettes were 1 cm pathlength quartz cells and nitrogen was continuously circulated through the cuvette compartment. Prior to the experiments, 3 μM solutions of the oligomers to be studied were mixed together in 1 M NaCl, 0.01 M sodium cacodylate (pH 7.0) or 0.1 M NaCl, 0.01 M sodium cacodylate (pH 7.0) and allowed to incubate at 90°C for a length of time sufficient to allow the optical density of the mixture to be perfectly stable (about 1 hour). Digitized absorbance and temperature values were stored in a computer for subsequent plotting and analysis. The temperature variation was $0.5°C.min^{-1}$. Prior to the recording of the data from the melting experiments (from 0°C up to 90°C) the mixture was the same as above.

Studies of the comparative stabilities of 4'-SU_6 8, 4'-SU_6-nprOH 10, and U_6 with respect to degradating enzymes

In a typical experiment, a solution of hexamer (1 A_{260}) dissolved in a suitable enzymatic buffer (1 ml) (Table 1) was mixed with an appropriate concentration of the corresponding enzyme (Table 1). Then aliquot fractions were incubated at 37°C for different periods of time before being analysed on a reverse phase HPLC apparatus using the 'on line ISRP cleaning' method [17]. The disappearance of the HPLC signal of the hexamer as a function of time was monitored and the corresponding kinetic constants calculated.

The same procedure was applied for the study of the stability of 4'-SU_6, **8**, in culture medium, except that the hexamer was directly dissolved in the commercial RPMI 1640 + 10% fetal calf serum used as the culture medium (1 ml).

Fluorescence experiments

In experiments using the intrinsic tryptophan fluorescence of RT, the excitation was performed at 295 nm in a quartz cuvette with a 5 mm optical path length to reduce the oligonucleotide inner filter effect. The emission intensity was measured at 338 nm, and the spectra were corrected for the buffer blank. The enzyme concentration was in the range of 4 to 12 nM. The fluorescent 36/19 DNA/DNA duplex, (36.F 19), used in displacement

5' TCCCTGTTCGGGCGCCAC 3' 11

3' AGGGACAAGCCCGCGGTGACGATCTCTAAAAGGTGT 5'

Figure 1. 36/18 duplex.

reactions was prepared by labelling the 36/18 duplex **11** (fig. 1) with succinylfluorescein dideoxy TTP in the presence of RT, as already described [21].

In displacement experiments of 36.F 19, the excitation was performed at 500 nm and the fluorescence emission intensity recorded at 532 nm. The experimental procedure was the same as previously published [21]. The titration started with a 1: 1 complex of RT and 36.F 19, and the total enzyme concentration was in the range of 12 to 25 nM.

The data from both types of experiments were transfered to a computer and evaluated by the use of the fitting program Grafit (Erithacus Software LTD.). In the case of decreasing fluorescence intensity, the following equation was used:

$$F = Fmax - \{(Et + L + K_D) - [(Et + L + K_D)^2 - 4Et.L]^{1/2}.(Fmax - Fmin)\}/2\ Et,$$

where F is the observed fluorescence intensity, Fmax is the fluorescence intensity at the start of the titration and Fmin at saturating concentration of the ligand; Et is the total concentration of the enzyme, L the concentration of the ligand, and K_D the dissociation constant of the enzyme/ligand complex.

In experiments in which 36.F 19 was displaced from its complex with RT by titration with unlabelled template.primer analog or primer analog alone, the observed fluorescence changes were described using the following equation:

$$F = Fmax - \{(Fmax - Fmin)[(KrE_0 - KrP_{T2}) - ((KrE_0 + KrP_{T2})^2 - 4(Kr - 1)KrE_0P_{T2})^{1/2}]\}/E_0(Kr - 1)$$

where F is the observed fluorescence intensity, Fmax and Fmin are the fluorescence intensities at the beginning of the titration and at saturating concentration of nonfluorescent ligand (P_{T2}), respectively. Kr is the relative affinity for RT of the non fluorescent ligand compared to the fluorescent ligand ($Kr = K_1/K_2$ where K_1 is the dissociation constant of the complex: fluorescent ligand/RT and K_2 the dissociation constant of the non fluorescent ligand complex with RT).

RESULTS AND DISCUSSION

One of the most powerful strategies to synthesize oligoribonucleotides is the use of the solid phase technique in combination with the phosphoramidite methodology [22]. Application of this strategy for 4'-thio-β-oligoribonucleotide synthesis requires the preparation of suitable protected 4'-thio-β-ribonucleoside phosphoramidites **5** (figure 2).

After preparing the 4'-thio-β-D-uridine **1** [14] and protecting the 5'-OH function with the DmTr group, we reacted the corresponding derivative **2** with *tert*-butyldimethylsilyl chloride (TBDMSCl) in pyridine in the presence of imidazole [23]. As expected, a mixture of 2' and 3' TBDMS derivatives **3** and **4** were obtained in a 2/1 ratio with a total yield of 39%. To enhance the 2'-O-TBDMS derivative yield, we applied the more recent Ogilvie procedure [24]. By reacting **2** with TBDMSCl in THF

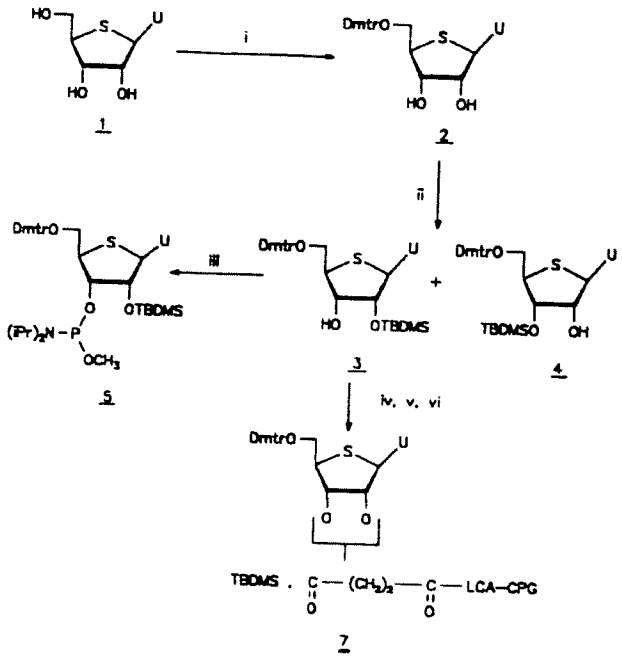

Figure 2. Synthesis of protected 4'-thio-β-D-uridine phosphoramidite, **5**, and derivatised solid support, **7**,. i = Dimethoxytrityl chloride, pyridine; ii = *tert*-butyldimethylsilyl chloride, imidazole, pyridine; iii = 4-dimethylaminopyridine, diisopropylethylamine, methylene chloride, (N,N-diisopropylamino) methyl phosphonamidic chloride, iv = succinic anhydride/4-dimethyl aminopyridine/triethylamine/methylene chloride; v = pentachlorophenol/1, 3-di cyclohexyl carbodiimide/1, 2-dimethoxyethane; vi = long chain alkylamine controlled pore glass/pyridine and then acetic anhydride/2, 6-lutidine/4-dimethylaminopyridine/tetrahydrofuran.

in the presence of $AgNO_3$ and pyridine, 2'-O-TBDMS **3** and 3'-O-TBDMS **4** were recovered in the same ratio but the overall yield (83%) was largely improved. These two isomers were then separated by silica gel chromatography and fully characterized by ^{1}H-NMR spectroscopy decoupling experiments. Finally, the methoxyphosphoramidite **5** was synthesized (yield 78%) according to a procedure similar to that already reported for the synthesis of β-ribonucleoside phosphoramidites in the oxygenated series [20]. As expected, the ^{31}P-NMR spectrum of **5** exhibited only one pair of signals for each diastereoisomeric (Rp and Sp) 4'-thio-β-ribonucleoside phosphoramidites. These results indicate that the annular sulfur atom in the sugar moiety does not hamper the synthetic procedure to obtain phosphoroamidites synthons as compared to the ribofuranose series.

As a first example of solid phase phosphoroamidite synthesis of 4'-thio-β-D-oligoribonucleotides, we prepared 4'-S(U-U-U-U-U-U), (4'-SU_6), **8** and 4'-S(U-U-U-U-U-U-U-U-U-U-U-U), (4'-SU_{12}), **9**. The solid support **7** necessary for the synthesis of **8** and **9** was obtained *via* the formation of a succinyl linkage between the 2'-hydroxyl group of 3'-TBDMS-4'-thio-β-uridine **4** and the amino function of long chain alkylamines on controlled pore glass beads (LCA-CPG). These oligomers were assembled automatically on a DNA synthesizer. As in the oxygenated β [20] or α series [6, 7], the condensation time required to obtain high coupling yields (> 98% as determined from the dimethoxytrityl cation release) was 15 min using 0.5 M tetrazole as activator

Table 2. Half-life time (min) of oligoribonucleotides U_6 and 4'-SU_6 in the presence of various nucleases.

	Half-Life Time (min)	
Nucleases	U_6	4'-SU_6
5'-Exonuclease Calf spleen phosphodiesterase	17	3900
3'-Exonuclease Snake venom phosphodiesterase	1	76
Endonuclease S_1	120	930
Ribonuclease A	<1	670

and 0.1 M phosphoramidite **5** in acetonitrile. The 4'-thio-oligomers were deprotected using a standard protocol and purified by reverse phase HPLC (spectrophotometric purities at 260 nm better than 98%).

The substrate activities of 4'-SU_6 **8**, with respect to various nucleases, was studied compared to the parent U_6. The enzymatic hydrolysis were monitored by HPLC by recording the disappearance of the signals corresponding to the starting oligomers. The observed results are reported in Table 2.

Compared to the wild type, 4'-SU_6 is much more resistant to the four considered degradating enzymes than U_6. Our results show that the most active nuclease is the snake venom phosphodiesterase in accordance with previous data [6]. It is well known that 3'-end protection of an oligomer confers an enhancement of the enzymatic stability towards 3'-exonucleases [17]. As an example, we synthesized 4'-SU_6-nprOH, **10**, in which a phosphodiester propanol linkage is introduced at the 3'-termini of the oligomer by means of the 'Universalis' solid support **6** [4]. This protected hexamer exhibited a half-life time of 250 min in the presence of snake venom phosphodiesterase. This result shows that the stability of the oligomer with respect to 3'-exonuclease can be improved by a factor 3 compared to the parent modified hexamer 4'-SU_6 under the same experimental conditions. Finally, the use of 4'-thio-oligomers greatly improved nuclease resistance compared to the corresponding oxygenated oligoribonucleotides.

We then evaluated the stability of 4'-SU_6, **8**, in cell culture medium at 37°C. RPMI 1640 complemented with 10% heat inactivated fetal calf serum was chosen because of its frequent use in hybrid translation arrest experiments *in vitro*. 'On line ISRP cleaning' HPLC [17] monitoring of the disappearance signals corresponding to the parent hexamer and its metabolites 4'-SU_5, 4'-SU_4 and 4'-SU_3 are shown in figure 3A assuming a preference for the 3'-termini enzymatic cleavage of the oligomers [25]. Only the kinetic curves of 4'-SU_6 and its first metabolite 4'-SU_5 are represented in figure 3B.

Treatment of kinetics data was by means of curvilinear regression according to the simple model: 4'-SU_6 $\xrightarrow{k1}$ 4'-SU_5 $\xrightarrow{k2}$ 4'-SU_4, and was in accordance with a first-order mechanism for the transformation of 4'-SU_6 with two step consecutive first order kinetics for the first metabolite 4'-SU_5. The hydrolysis rate constants k_1 and k_2 thus obtained are presented on table 3 along with the corresponding degradation half-live times.

Enzymatic cleavages of 4'-SU_6 and its first metabolite 4'-SU_5 exhibit similar hydrolysis rate constants (Table 3). These results suggest that consecutive decomposition in culture medium is

Table 3. Hydrolysis rate constants and half-life time of 4'-SU_6 and 4'-SU_5 in culture medium.

Oligonucleotide	Hydrolysis rate constant (min^{-1})	Half-life time (min)
4'-SU_6	$3.80.10^{-3}$	182
4'-SU_5	$3.49.10^{-3}$	198

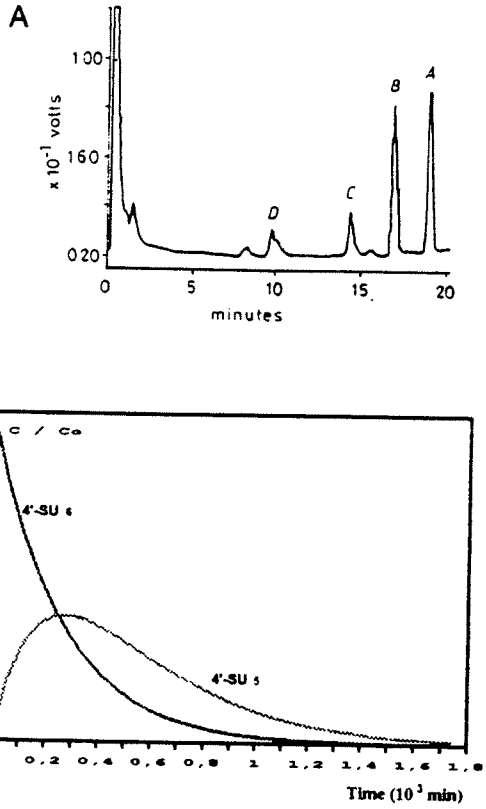

Figure 3.A. Reverse-phase HPLC (260 nm) profiles of 4'-SU_6 after 4 hrs incubation in culture medium RPMI 1640 + 10% heat inactivated fetal calf serum. A: 4'-SU_6, B: 4'-SU_5, C: 4'-SU_4, D: 4'-SU_3. **B.** Kinetics of enzymatic degradation of 4'-SU_6 and 4'-SU_5 in culture medium containing RPMI 1640 and 10% heat inactivated fetal calf serum.

controlled by a 3'-exonuclease activity and corroborate previous reports indicating that in serum [26] and in plasma [27], 3'-exophosphodiesterases were largely involved in the breakdown of oligomers. In agreement with Hoke's and Eder's [26, 27] results, neither endonuclease nor 5'-exonuclease activities were detected, thereby confirming that heat treatment (65°C, 30 min) of the fetal calf serum inactivates such enzymatic activities. Due to their high nuclease resistance, it is noteworthy that all the sterile conditions required during the handling and the storage of natural RNA are not necessary for 4'-S-RNA. 4'-thio-oligomers were recovered intact after being stored frozen for several months at −20°C.

Table 4. Tm values of 4'-SU_{12} hybrids with complementary RNA or DNA strands.

Duplex	[NaCl]	Tm °C
4'-SU_{12} / Poly(A)	1 M	46
4'-SU_{12} / d($C_2A_{12}C_2$)	1 M	27
4'-SU_{12} / Poly(A)	0.1M	33
4'-SU_{12} / d($C_2A_{12}C_2$)	0.1M	5

We also determined the capacity of the 4'-thiododecamer 4'-SU_{12}, **9**, to form stable duplexes with complementary RNA and DNA sequences. Base pairing between 4'-SU_{12}, **9**, and its complementary d($C_2A_{12}C_2$) or Poly(A) strands was studied by UV absorption spectroscopy. Upon increasing the temperature of a pre-cooled equimolar 4'-SU_{12}/Poly(A) or 4'-SU_{12}/d($C_2A_{12}C_2$) mixture, hyperchromicity was observed from which a melting temperature was determined (Table 4). Allowing the temperature to slowly decrease, we did not observe any hysteresis phenomena. On the contrary, a clear transition step occured with the same Tm value as in the melting experiment. A comparison of the Tm data obtained from melting curves at high (1 M NaCl) and low (0.1 M NaCl) salt concentrations confirms the importance of ionic strength on the duplex stability of charged oligomers.

The Tm data in table 4 indicate that the homoduplex 4'-SU_{12}/Poly(A) is more stable than the heteroduplex 4'-SU_{12}/d($C_2A_{12}C_2$) at the two salt concentrations investigated. The Tm value (46°C) determined for the homoduplex 4'-SU_{12}/Poly(A) is larger than the calculated melting temperature of the duplex U_{12}/A_{12} (Tm = 32°C, 1 M NaCl, concentration of oligonucleotide: 3 μM) [28] suggesting that the introduction of a sulfur atom in the 4'-thio oligoribonucleotide improved its thermal stability in duplexes compared to the natural product. Also, noteworthy are the Tm values obtained for both duplexes at near physiological conditions (0.1 M NaCl): we observe that a strong destabilisation occurs for the heteroduplex 4'-SU_{12}/d($C_2A_{12}C_2$) at this low salt concentration whereas the homoduplex 4'-SU_{12}/Poly(A) is still stable under the same conditions (Table 4). This is in accordance with previous data [29, 30] reporting that RNA/RNA duplexes are more stable than RNA/DNA duplexes. It must be kept in mind that the overwhelming majority of antisense molecules are designed to exert their effect upon hybridisation to RNA species. The Tm value (33°C) observed under nearly physiological conditions for the homoduplex 4'-SU_{12}/Poly(A) in conjunction with the high nuclease resistance properties of 4'-S-RNA suggest that this new 4'-S-RNA series is a good candidate for providing potential antisense effects.

Homopolymeric oligonucleotide analogs have been successfully used as non sequence specific inhibitors of HIV-1 reverse transcriptase (RT). Phosphorothioate oligodeoxynucleotides [31] or α-oligonucleotides [32], either annealed or non annealed to a template, competitively inhibit the incorporation of dTMP at the 3'-end of dT_{14} bound to Poly(A) [33, 34]. The study of the direct interaction with RT of these oligonucleotide analogs is now readily accessible by fluorescence spectrometry [18]. The resulting dissociation constants K_D express the affinities of these oligomers for the enzyme and pertain to the initially formed complex, in the absence of any substrate. Therefore, comparison

Table 5. Interaction of 4'-SU_6, **8**, and 4'-SU_{12}, **9**, with HIV-1 RT: Dissociation constants of complexes as determined by fluorescence change measurements at 25°C.

Ligand	Intrinsic fluorescence		Displacement of fluorescent 36.F19 Kd (nM)
	Kd (nM)[a]	ΔF %[b]	
4'-SU_6	4 ± 0.5 (4.2)[d]	19	c
Poly(A).4'-SU_6	14.5 ± 0.5 (13)[d]	27	260
4'-SU_{12}	11 ± 1.2 (12.2)[d]	21	c
Poly(A).4'-SU_{12}	19 ± 4 (18.5)[d]	29	310
Hybrid 36 / 18	1.8	25	-
Hybrid 36 / 18 in the presence of 4'-SU_6	2.1	25	-

a) Average value from 4 experiments. Relative experimental errors were estimated to be less than 10%. b) ΔF% = (Fmax − Fmin) 100/F max, where Fmax is the observed fluorescence intensity in the absence of ligand and Fmin the fluorescence intensity at saturating concentrations of ligand. c) No change in fluorescence intensity. d) Values between brackets are the Kd determined in the presence of saturating concentrations of **11**.

of K_D constants to K_i constants determined from steady-state kinetics can be expected to provide information about the reaction mechanism. To date, very few RNA homopolymeric oligonucleotide primer analogs have been studied as possible negative effectors of RT activity, even though the natural primer is t-RNA Lys 3 [35]. Accordingly, we have studied the interaction of the new oligonucleotide analogs **8** and **9** with RT. The affinity for RT of **8** and **9** or of the corresponding hybrids with Poly(A) were first determined using two different methods based on fluorescence intensity changes upon complexation of the ligands to the enzyme. The first method uses the intrinsic fluorescence of the protein due to the 37 tryptophan residues in the p66.p51 heterodimeric form of the enzyme [18, 36]. Excitation at 295 nm and fluorescence emission intensity scanned at 315 and 400 nm during the titration of RT with the oligomers **8** or **9** and the hybrids with Poly(A) gave the results reported in table 5. Both oligomers **8** and **9** induced significant intrinsic fluorescence quenching and showed a marked affinity for the enzyme, the 6-mer **8** giving the most stable complex, contrary to phosphorothioate oligonucleotides where the affinity increases with chain length [33]. The corresponding hybrids with Poly(A) have slighly lower affinities and their complexation produces a larger quenching of fluorescence than the non annealed oligomers.

In a second set of experiments, a fluorescent DNA/DNA duplex ligand was used as a probe in displacement reactions with **8** and **9** or their hybrids. This fluorescent probe was prepared by RT catalysed incorporation of succinylfluorescein-labelled dideoxy TTP to the 3'-end of a 36/18 DNA/DNA duplex **11** yielding a 36/19 fluorescent hybrid (36.F 19) [21]. Consequently, both **11** or 36.F 19 bind the enzyme at the polymerase active site. Addition of **8** or **9** up to a concentration of 250 nM to a solution of a 1: 1 complex between the enzyme and 36.F 19 gave no change in the intensity of the fluorescence emission at 532 nm. Since the release of 36.F 19 from its complex with RT results in a reduction of fluorescence by a factor of 2 [21], there is no significant competition between 36.F 19 and **8** and **9** for binding to the active site of RT over a 30 min time period. This suggests that **8** and **9** bind the enzyme at a distinct secondary site. The same competition experiments with **8** and **9** annealed to Poly(A) led to displacement of 36.F 19 from its complex only when relatively high concentrations of non fluorescent ligand were used.

The high K_D values (Table 5) are consistent with very low binding affinities of the hybrids for the polymerase site of RT. We also investigated the effect of **8** and **9** on the change in intrinsic fluorescence intensity of RT during its titration with the non fluorescent 36/18 hybrid **11**. The presence of **8** and **9** at saturating concentration prior to the start of the titration changed neither the value of the dissociation constant of the complex between the enzyme and the 36/18 hybrid nor the level of fluorescence quenching induced by the binding. Alternatively, the presence of saturating concentrations of **11** did not change the affinities of **8**, **9** or their hybrids for RT as determined from change in intrinsic fluorescence of the enzyme (Table 5).

The steady state kinetics of the interaction with RT of **9** or its hybrid with Poly(A) have also been studied. In the presence of concentrations of **9** annealed to Poly(A) in the range of 0 to 1 μM and 20 μM dTTP, no priming of the polymerisation by **9** was observed. In the same conditions and with Poly(A)/dT_{14} as template primer, neither **9** nor its duplex with Poly(A) inhibited the enzyme. On the contrary, Poly(A)/4′-SU_{12} induced a small activation (5−10%) of dTTP incorporation while the addition of an equivalent quantity of the template alone had practically no effect. Addition of nonannealed **9** decreased slightly the rate of polymerisation, but the pattern of the inhibition (as in the results reported in reference 34) suggests that **9** occupies some portion of free stretches of the template downstream from the primer, thereby blocking polymerisation without interacting directly with the enzyme. This is consistent with the fact that the binding of **8**, **9** or their hybrids in the secondary site has no effect on the binding of **11** or 36/18 in the active site, or reciprocally.

In conclusion, we have synthesized for the first time two homo-4′-thio-oligoribonucleotides, namely 4′-SU_6, **8**, and 4′-SU_{12}, **9**. We have shown that such oligomers possess a high nuclease resistance as compared to the wild type RNA. In addition, we studied their capacity to bind to complementary nucleic acid sequences and we found that they can form stable duplexes with Poly(A) under nearly physiological conditions. In contrast, a strong destabilisation effect was noticed when these oligomers were associated with the corresponding DNA. With respect to the interaction of these oligomers with reverse transcriptase, all available evidence is consistent with the existence of binding to RT of **8** and **9**, annealed or non annealed to Poly(A), at a site which is different from the polymerase site of the enzyme. The binding of the oligomers to this secondary site does not inhibit DNA polymerisation catalysed by the enzyme.

All these data firmly indicate that this new stable chimeric RNA series of β configuration merits further study to evaluate its capacity to interfere with the nucleic acid cellular machinery.

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